INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ^{6} :		(11) International Publication Number:	**WO 97/06272**
C12N 15/86, C07K 14/015, C12N 5/10, 7/04	A2	(43) International Publication Date:	20 February 1997 (20.02.97)

(21) International Application Number: PCT/US96/12751	(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
(22) International Filing Date: 1 August 1996 (01.08.96)	
(30) Priority Data: 08/510,790 3 August 1995 (03.08.95) US 08/688,648 29 July 1996 (29.07.96) US	**Published** *Without international search report and to be republished upon receipt of that report.*
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(54) Title: HIGH EFFICIENCY HELPER SYSTEM FOR AAV VECTOR PRODUCTION
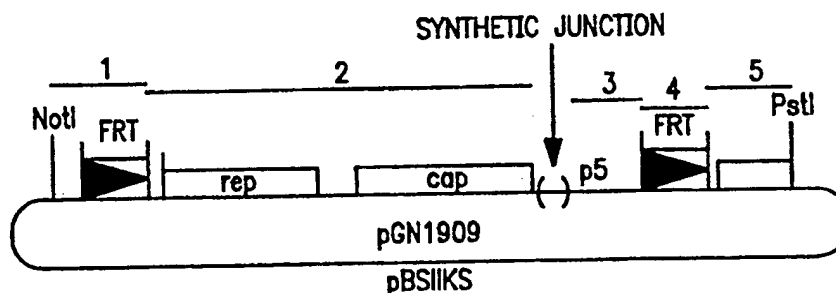
(57) Abstract

Novel nucleic acid molecules are provided having adeno-associated virus (AAV) coding regions that are capable of expressing necessary AAV functions to complement an AAV vector in the production of recombinant AAV (rAAV) virions. The molecules feature a nucleotide sequence that is substantially homologous to an AAV p5 promoter region, wherein the p5 promoter region is situated in the molecules in a site that is other than its natural position relative to the AAV *rep* coding region in the wild-type AAV genome. AAV helper function constructs are also provided, comprising the instant nucleic acid molecules embodied in a replicon that is capable of being transcribed and translated to express complementing AAV helper functions in a suitable host cell. Novel AAV packaging cells and AAV producer cells are provided, which contain the AAV helper constructs of the invention, and methods of producing enhanced levels of rAAV virions using the AAV helper constructs of the invention are also provided. Methods are also provided for producing rAAV virions without the concomitant production of significant levels of wild-type AAV.

5   HIGH EFFICIENCY HELPER SYSTEM
              FOR AAV VECTOR PRODUCTION

                    Description

10

Technical Field

        The present invention relates generally to
helper function systems for use in adeno-associated
virus (AAV) vector production. More specifically, the
15   invention relates to AAV helper function constructs
which provide for the expression of essential AAV *rep*
and *cap* functions necessary for production of AAV
virions.

20   Background of the Invention

        Gene delivery is a promising method for the
treatment of acquired and inherited diseases. A
number of viral based systems for gene transfer
purposes have been described, such as retroviral
25   systems which are currently the most widely used viral
vector systems for this purpose. For descriptions of
various retroviral systems, *see*, *e.g.*, U.S. Patent No.
5,219,740; Miller and Rosman (1989) *BioTechniques*
7:980-990; Miller, A.D. (1990) *Human Gene Therapy* 1:5-
30   14; Scarpa et al. (1991) *Virology* 180:849-852; Burns
et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:8033-8037;
and Boris-Lawrie and Temin (1993) *Cur. Opin. Genet.
Develop.* 3:102-109.

        Adeno-associated virus (AAV) systems have
35   also been used for gene delivery. AAV is a helper-
dependent DNA parvovirus which belongs to the genus
Dependovirus. AAV requires co-infection with an

unrelated helper virus, either adenovirus, a
herpesvirus or vaccinia, in order for a productive
infection to occur. In the absence of such co-
infection, AAV establishes a latent state by insertion

5   of its genome into a host cell chromosome. Subsequent
infection by a helper virus rescues the integrated
copy which can then replicate to produce infectious
viral progeny. . AAV has a wide host range and is able
to replicate in cells from any species so long as

10   there is also a successful co-infection of such cells
with a suitable helper virus. Thus, for example,
human AAV will replicate in canine cells co-infected
with a canine adenovirus. AAV has not been associated
with any human or animal disease and does not appear

15   to alter the biological properties of the host cell
upon integration. For a review of AAV, see, e.g.,
Berns and Bohenzky (1987) *Advances in Virus Research*
(Academic Press, Inc.) 32:243-307.
             The AAV genome is composed of a linear,

20   single-stranded DNA molecule which contains 4681 bases
(Berns and Bohenzky, *supra*). The genome includes
inverted terminal repeats (ITRs) at each end which
function in *cis* as origins of DNA replication and as
packaging signals for the virus. The ITRs are

25   approximately 145 bp in length. The internal
nonrepeated portion of the genome includes two large
open reading frames, known as the AAV *rep* and *cap*
regions, respectively. These regions code for the
viral proteins involved in replication and packaging

30   of the virion. In particular, a family of at least
four viral proteins are synthesized from the AAV *rep*
region, Rep 78, Rep 68, Rep 52 and Rep 40, named
according to their apparent molecular weight. The AAV
cap region encodes at least three proteins, VP1, VP2

35   and VP3. For a detailed description of the AAV
genome, see, e.g., Muzyczka, N. (1992) *Current Topics
in Microbiol. and Immunol.* 158:97-129.

The construction of recombinant AAV virions has been described. *See, e.g.,* U.S. Patent Nos. 5,173,414 and 5,139,941; International Publication Numbers WO 92/01070 (published 23 January 1992) and WO

5   93/03769 (published 4 March 1993); Lebkowski et al. (1988) *Molec. Cell. Biol.* 8:3988-3996; Vincent et al. (1990) *Vaccines 90* (Cold Spring Harbor Laboratory Press); Carter, B.J. (1992) *Current Opinion in Biotechnology* 3:533-539; Muzyczka, N. (1992) *Current*

10   *Topics in Microbiol. and Immunol.* 158:97-129; and Kotin, R.M. (1994) *Human Gene Therapy* 5:793-801.

Recombinant AAV (rAAV) virions are generally produced in a suitable host cell that has been transfected with two constructs including an AAV

15   vector plasmid and a helper plasmid, whereby the host cell is thus capable of expressing the AAV proteins necessary for AAV replication and packaging (AAV helper functions). The host cell is then co-infected with an appropriate helper virus to provide necessary

20   viral helper functions. AAV helper functions can be provided by transfecting the host cell with an AAV helper plasmid that includes the AAV *rep* and/or *cap* coding regions but which lacks the AAV ITRs. Accordingly, the helper plasmid can neither replicate

25   nor package itself. A number of vectors that contain the *rep* coding region are known, including those vectors described in U.S. Patent No. 5,139,941, having ATCC accession numbers 53222, 53223, 53224, 53225 and 53226. Similarly, methods of obtaining vectors

30   containing the HHV-6 homologue of AAV *rep* are described in Thomson et al. (1994) *Virology* 204:304- 311. A number of vectors containing the *cap* coding region have also been described, including those vectors described in U.S. Patent No. 5,139,941.

35   Packaging cell lines derived from human 293 cells that have been transfected with a vector having the AAV *rep* gene operably linked to a heterologous transcription

-3-

promoter have been described in International
Publication Nos. WO 95/13392, published 18 May 1995,
and WO 95/13365, published 18 May 1995.

   5   In rAAV virion production, AAV vector
plasmids can be engineered to contain a functionally
relevant nucleotide sequence (e.g., a selected gene,
antisense nucleic acid molecule, ribozyme, or the
like) of interest that is flanked by AAV ITRs which
provide for AAV replication and packaging functions.
  10 Both AAV helper plasmids and the AAV vector plasmid
bearing the nucleotide sequence are introduced into
recipient cells by transient transfection. The
transfected cells are then infected with adenovirus
which transactivates the AAV promoters present on the
  15 helper plasmid that direct the transcription and
translation of AAV *rep* and *cap* regions. rAAV virions
harboring the nucleotide sequence of interest are
formed and can be purified from the preparation.

             A host cell that has been transfected with a
  20 helper plasmid encoding AAV helper functions comprises
a packaging cell which, by virtue of the transfection,
is capable of expressing AAV gene products to
complement necessary functions deleted from a selected
AAV vector plasmid. A number of attempts have been
  25 made to establish packaging cell systems.
Particularly, Mendelson et al. (1988) *Virology*
<u>166</u>:154-165 reported a cell line capable of low level
expression of one of the short forms of Rep using
stable transfection of HeLa or 293 cells with plasmids
  30 containing the *rep* gene. Cell lines containing
integrated AAV *rep* and *cap* genes expressed from the
normal AAV promoters have also been described.
Vincent et al. (1990) *Vaccines 90*, Cold Spring Harbor
Laboratory Press, pp. 353-359.

  35   Other approaches have attempted to establish
packaging cell line systems containing AAV vectors,
either stably integrated into the host cell genome, or

maintained as an episomal plasmid. The cell lines are then transfected with *trans* complementing AAV functions such as with constructs containing the AAV *rep*, or *rep* and *cap* genes. *See, e.g.*, U.S. Patent No.

5   5,173,414 to Lebkowski, and International Publication Nos. WO 95/13365, published 18 May 1995 and WO 95/13392, published 18 May 1995.

However, a number of problems have been encountered in the aforementioned packaging cell

10   systems which have greatly limited their utility. Particularly, such systems have not been able to generate significant levels of recombinant AAV virions. Not being bound by any particular theory, such problems may be due in part to several inhibitory

15   effects attributed to expressed *rep* gene products in those cell systems. *See, e.g.*, Labow et al. (1987) *Mol. Cell. Biol.* 7:1320-1325 and Tratschin et al. (1986) *Mol. Cell. Biol.* 6:2884-2894. Another problem has been the production of significant levels of

20   contaminating wild-type AAV particles (e.g., replication-competent AAV particles) in such packaging cell systems due to recombination events between AAV vector and helper plasmid sequences. Senapathy et al. (1984) *J. Biol. Chem.* 259:4661-4666.

25   Accordingly, there remains a need to provide improved AAV helper function constructs that are capable of being expressed in a host cell at efficient levels. Further, there remains a need to provide AAV packaging cell systems capable of producing

30   commercially significant levels of recombinant AAV particles without also generating significant levels of contaminating recombined wild-type AAV particles.

Summary of the Invention

35   The present invention provides for novel nucleic acid molecules having nucleotide sequences that are substantially homologous to AAV p5 promoter

regions and AAV coding regions (e.g., AAV *rep* and *cap*
coding regions). The p5 promoter region can be
situated in the nucleic acid molecules in a site other
than its normal position relative to the AAV *rep*
5   coding region in the wild-type AAV genome. The
subject molecules, having a p5 promoter region which
has been effectively moved from its normal upstream
position (relative to the *rep* coding region in the
wild-type (wt) AAV genome) exhibit several novel
10   features. First, relocation of the p5 promoter region
may result in an attenuation of the production of at
least the long form Rep products when the *rep* coding
region is expressed. Further, the *cis* acting
functions necessary for expression from the AAV p19
15   and p40 promoters are present such that *rep52/40* and
cap appear to be normally expressed. Also, in
particular molecules, the unwanted generation of
contaminating wild-type AAV particles during rAAV
production can be reduced or eliminated.
20   Also provided herein are nucleic acid
molecules having AAV coding regions (e.g., AAV *rep* and
cap coding regions) and a nucleotide sequence that
comprises an AAV p5 promoter region, wherein the
nucleotide sequence is arranged in the molecule such
25   that the p5 promoter is situated in a site other than
its normal upstream position relative to the AAV *rep*
coding region in the wt AAV genome.
           The above-described nucleic acid molecules
can further include one, or a plurality of additional
30   nucleotide sequences--wherein the single additional
nucleotide sequence is arranged 5' to the AAV coding
regions and the p5 promoter region, or the plurality
of additional nucleotide sequences are arranged to
flank the AAV coding regions and the p5 promoter
35   region. These additional nucleotide sequences are
substantially homologous to yeast FLP recombinase
substrates (e.g., Flip Recombination Target (FRT)

sites). Provision of 5' and 3' flanking FRT sites allows for the excision of the nucleic acid molecule from a vector construct by the action of the FLP recombinase enzyme.

5   The present invention also provides for AAV helper constructs that are capable of being expressed to provide AAV Rep and Cap polypeptides. Such helper constructs can be formed by operably linking the nucleic acid molecules of the invention to suitable

10   control elements that are capable of directing the transcription and translation of the AAV coding regions contained in the constructs. The AAV helper constructs provided herein can comprise plasmids or any other suitable vector, and can further be

15   constructed to include selectable genetic markers such as antibiotic resistance genes or the like. In one particular embodiment, the AAV helper construct comprises the plasmid pGN1909 (ATCC Accession Number 69871). In other particular embodiments, the AAV

20   helper construct comprises either the plasmid pW1909-1 or the plasmid pH8.

          The invention further provides for AAV packaging cells that are capable of becoming AAV producer cells when an AAV vector is present therein

25   and the packaging cell is capable of expressing viral helper functions. The subject AAV packaging cells are produced by introducing the AAV helper constructs of the present invention into a suitable host cell. More particularly, the helper constructs can be either

30   transiently or stably transfected into suitable host cells using known techniques.

          Also provided herein are AAV producer cells that are capable of producing rAAV virions when viral helper functions are expressed therein. The subject

35   producer cells are formed by transfection of the AAV packaging cells of the present invention with a suitable AAV vector. In accordance with the

invention, the AAV vector generally comprises a
heterologous nucleotide sequence that is flanked by
functional AAV ITRs. The production of rAAV virions
that contain the heterologous nucleotide sequence (for
5   subsequent transduction) can be accomplished by
introducing viral helper functions into the producer
cells to transactivate the AAV helper functions
present in the AAV helper constructs.

The invention further provides methods of
10   producing rAAV virions which include the steps of:
introducing an AAV vector into a suitable host cell;
introducing an AAV helper construct selected from
those provided herein into the host cell to express
essential AAV helper functions; expressing viral
15   helper functions in the host cell; and culturing the
cell to produce rAAV virions. The AAV vector and AAV
helper constructs can be transfected into the host
cell, either sequentially or simultaneously, using
techniques known to those of skill in the art. The
20   expression of viral helper functions can be provided
by infecting the host cell with a suitable helper
virus selected from the group of adenoviruses,
herpesviruses and vaccinia viruses. The viral helper
functions transactivate AAV promoters present in the
25   AAV helper construct that direct the transcription and
translation of AAV *rep* and *cap* regions. Thus, rAAV
virions harboring a selected heterologous nucleotide
sequence are formed and can be purified from the
preparation using known methods.
30   These and other embodiments of the subject
invention will readily occur to those of ordinary
skill in the art in view of the disclosure herein.

Brief Description of the Figures
35   Figure 1 depicts the construction of the
pGN1909 plasmid construct.

Figure 2 is a representation of plasmid
pGN1909.

Figure 3 shows the nucleotide sequences of a
76 bp FRT site [SEQ ID NO:3] and a 59 bp "minimum" FRT
5   site [SEQ ID NO:2] that are useful in the construction
of the AAV helper constructs of the present invention.

Figure 4 depicts a polynucleotide sequence
consisting of base pairs 145 through 494 [SEQ ID NO:4]
of the wild-type AAV serotype 2 genome.

10

Detailed Description of the Invention

The practice of the present invention will
employ, unless otherwise indicated, conventional
methods of virology, microbiology, molecular biology
15   and recombinant DNA techniques within the skill of the
art. Such techniques are explained fully in the
literature. See, e.g., Sambrook, et al. Molecular
Cloning: A Laboratory Manual (Current Edition); DNA
Cloning: A Practical Approach, vol. I & II (D.
20   Glover, ed.); Oligonucleotide Synthesis (N. Gait, ed.,
Current Edition); Nucleic Acid Hybridization (B. Hames
& S. Higgins, eds., Current Edition); Transcription
and Translation (B. Hames & S. Higgins, eds., Current
Edition); CRC Handbook of Parvoviruses, vol. I & II
25   (P. Tijessen, ed.); Fundamental Virology, 2nd Edition,
vol. I & II (B.N. Fields and D.M. Knipe, eds.)

All publications, patents and patent
applications cited herein, whether supra or infra, are
hereby incorporated by reference in their entirety.

30   As used in this specification and the
appended claims, the singular forms "a," "an" and
"the" include plural references unless the content
clearly dictates otherwise.

35

-9-

A.   Definitions

        In describing the present invention, the
following terms will be employed, and are intended to
be defined as indicated below.

5   "Gene transfer" or "gene delivery" refers to
methods or systems for reliably inserting foreign DNA
into host cells. Such methods can result in transient
expression of non-integrated transferred DNA,
extrachromosomal replication and expression of

10   transferred replicons (e.g., episomes), or integration
of transferred genetic material into the genomic DNA
of host cells. Gene transfer provides a unique
approach for the treatment of acquired and inherited
diseases. A number of systems have been developed for

15   gene transfer into mammalian cells. *See, e.g.*, U.S.
Patent No. 5,399,346.

        By "vector" is meant any genetic element,
such as a plasmid, phage, transposon, cosmid,
chromosome, virus, virion, etc., which is capable of

20   replication when associated with the proper control
elements and which can transfer gene sequences between
cells. Thus, the term includes cloning and expression
vehicles, as well as viral vectors.

        By "adeno-associated virus inverted terminal

25   repeats" or "AAV ITRs" is meant the art-recognized
palindromic regions found at each end of the AAV
genome which function together in *cis* as origins of
DNA replication and as packaging signals for the
virus. AAV ITRs, together with the AAV *rep* coding

30   region, provide for the efficient excision and rescue
from, and integration of a nucleotide sequence
interposed between two flanking ITRs into a mammalian
cell genome.

        The nucleotide sequences of AAV ITR regions

35   are known. See, e.g., Kotin, R.M. (1994) *Human Gene*
Therapy 5:793-801; Berns, K.I. "Parvoviridae and their
Replication" in *Fundamental Virology*, 2nd Edition,

(B.N. Fields and D.M. Knipe, eds.) for the AAV-2
sequence. As used herein, an "AAV ITR" need not have
the wild-type nucleotide sequence depicted, but may be
altered, e.g., by the insertion, deletion or

5   substitution of nucleotides. Additionally, the AAV
ITR may be derived from any of several AAV serotypes,
including without limitation, AAV-1, AAV-2, AAV-3,
AAV-4, AAV-5, AAVX7, etc. Furthermore, 5' and 3' ITRs
which flank a selected nucleotide sequence in an AAV

10   vector need not necessarily be identical or derived
from the same AAV serotype or isolate, so long as they
function as intended, i.e., to allow for excision and
rescue of the sequence of interest from a host cell
genome or vector, and to allow integration of the

15   heterologous sequence into the recipient cell genome
when the *rep* gene is present in the cell (either on
the same or on a different vector).

         By an "AAV vector" is meant a vector derived
from an adeno-associated virus serotype, including

20   without limitation, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5,
AAVX7, etc. AAV vectors can have one or more of the
AAV wild-type genes deleted in whole or part,
preferably the *rep* and/or *cap* genes, but retain
functional flanking ITR sequences. Functional ITR

25   sequences are necessary for the rescue, replication
and packaging of the AAV virion. Thus, an AAV vector
is defined herein to include at least those sequences
required in *cis* for replication and packaging (e.g.,
functional ITRs) of the virus. The ITRs need not be

30   the wild-type nucleotide sequences, and may be
altered, e.g., by the insertion, deletion or
substitution of nucleotides, so long as the sequences
provide for functional rescue, replication and
packaging.

35   AAV vectors can be constructed using
recombinant techniques that are known in the art to
include one or more heterologous nucleotide sequences

flanked on both ends (5' and 3') with functional AAV

ITRs. In the practice of the invention, an AAV vector

can include at least one AAV ITR and a suitable

promoter sequence positioned upstream of the

5   heterologous nucleotide sequence and at least one AAV

ITR positioned downstream of the heterologous

sequence. The 5' and 3' ITRs need not necessarily be

identical or derived from the same AAV isolate, so

long as they function as intended.

10   The selected heterologous nucleotide

sequence included in the AAV vector can comprise any

desired gene that encodes a protein that is defective

or missing from a recipient cell genome or that

encodes a non-native protein having a desired

15   biological or therapeutic effect (e.g., an antiviral

function), or the sequence can correspond to a

molecule having an antisense or ribozyme function.

Suitable genes include those used for the treatment of

inflammatory diseases, autoimmune, chronic and

20   infectious diseases, including such disorders as AIDS,

cancer, neurological diseases, cardiovascular disease,

hypercholestemia; various blood disorders including

various anemias, thalasemias and hemophilia; genetic

defects such as cystic fibrosis, Gaucher's Disease,

25   adenosine deaminase (ADA) deficiency, emphysema, etc.

A number of antisense oligonucleotides (e.g., short

oligonucleotides complementary to sequences around the

translational initiation site (AUG codon) of an mRNA)

that are useful in antisense therapy for cancer and

30   for viral diseases have been described in the art.

See, e.g., Han et al. (1991) *Proc. Natl. Acad. Sci.*

USA 88:4313-4317; Uhlmann et al. (1990) *Chem. Rev.*

90:543-584; Helene et al. (1990) *Biochim. Biophys.*

Acta. 1049:99-125; Agarwal et al. (1988) *Proc. Natl.*

35   *Acad. Sci. USA* 85:7079-7083; and Heikkila et al.

(1987) *Nature* 328:445-449. For a discussion of

suitable ribozymes, *see, e.g.,* Cech et al. (1992) *J.*

Biol. Chem. <u>267</u>:17479-17482 and U.S. Patent No.
5,225,347 to Goldberg et al.

AAV vectors can also include control
sequences, such as promoter and polyadenylation sites,
as well as selectable markers or reporter genes,
enhancer sequences, and other control elements which
allow for the induction of transcription. Such
control elements are described more fully below. Such
AAV vectors can be constructed using techniques well
known in the art. *See, e.g.,* U.S. Patent No.
5,173,414; International Publication Numbers WO
92/01070 (published 23 January 1992) and WO 93/03769
(published 4 March 1993); Lebkowski et al. (1988)
Molec. Cell. Biol. <u>8</u>:3988-3996; Vincent et al. (1990)
Vaccines 90 (Cold Spring Harbor Laboratory Press);
Carter, B.J. (1992) *Current Opinion in Biotechnology*
<u>3</u>:533-539; Muzyczka, N. (1992) *Current Topics in
Microbiol. and Immunol.* <u>158</u>:97-129; Kotin, R.M. (1994)
Human Gene Therapy <u>5</u>:793-801; Shelling and Smith
(1994) *Gene Therapy* <u>1</u>:165-169; and Zhou et al. (1994)
J. Exp. Med. <u>179</u>:1867-1875.

"AAV helper functions" refer to AAV-derived
coding sequences which can be expressed to provide AAV
gene products that, in turn, can function in *trans* for
productive AAV replication. Thus, AAV helper
functions include one, or both of the major AAV open
reading frames (ORFs)--*rep* and *cap*. The Rep
expression products have been shown to possess many
functions, including recognition, binding and nicking
of the AAV origin of DNA replication; DNA helicase
activity; and modulation of transcription from AAV (or
other heterologous) promoters. The Cap expression
products supply necessary packaging functions. AAV
helper functions are used herein to complement AAV
functions in *trans* that are missing from AAV vectors.

The term "viral helper functions" refers to
the provision of factors that are necessary during

-13-

various aspects of the AAV life cycle. AAV requires
such helper functions from an unrelated helper virus
(e.g., an adenovirus, a herpesvirus or a vaccinia
virus), in order for a productive AAV infection to

5   occur. Particularly, it has been demonstrated that
adenovirus supplies factors required for AAV promoter
expression, AAV messenger RNA stability and AAV
translation. *See, e.g.*, Muzyczka, N. (1992) *Curr.
Topics. Microbiol. and Immun.* 158:97-129. In the

10   absence of such functions, AAV establishes a latent
state by insertion of its genome into a host cell
chromosome. Production of viral helper functions
rescues the integrated copy which can then replicate
to produce infectious viral progeny. Viral helper

15   functions can be provided by infection of a cell with
a suitable helper virus.
        The term "AAV helper construct" refers
generally to a nucleic acid molecule that includes
nucleotide sequences providing AAV functions deleted

20   from an AAV vector which is to be used to produce a
transducing vector for delivery of a nucleotide
sequence of interest. AAV helper constructs are
commonly used to provide transient expression of AAV
rep and/or *cap* genes to complement missing AAV

25   functions that are necessary for lytic AAV
replication; however, helper constructs lack AAV ITRs
and can neither replicate nor package themselves. AAV
helper constructs can be in the form of a plasmid,
phage, transposon, cosmid, virus, or virion. A number

30   of AAV helper constructs have been described, such as
the commonly used plasmids pAAV/Ad and pIM29+45 which
encode both Rep and Cap expression products. *See,
e.g.*, Samulski et al. (1989) *J. Virol.* 63:3822-3828;
and McCarty et al. (1991) *J. Virol.* 65:2936-2945. A

35   number of other vectors have been described which
encode Rep and/or Cap expression products. *See, e.g.*,
U.S. Patent No. 5,139,941.

By "recombinant virus" is meant a virus that
has been genetically altered, e.g., by the addition or
insertion of a heterologous nucleic acid construct
into the particle.

5   By "AAV virion" is meant a complete virus
particle, such as a wild-type (wt) AAV virus particle
(comprising a linear, single-stranded AAV nucleic acid
genome associated with an AAV capsid protein coat), or
a recombinant AAV virus particle as described below.

10 In this regard, single-stranded AAV nucleic acid
molecules of either complementary sense, e.g., "sense"
or "antisense" strands, can be packaged into any one
AAV virion and both strands are equally infectious.

A "recombinant AAV virion," or "rAAV virion"

15 is defined herein as an infectious, replication-
defective virus composed of an AAV protein shell,
encapsidating a heterologous nucleotide sequence of
interest which is flanked on both sides by AAV ITRs.

The term "promoter region" is used herein in

20 its ordinary sense to refer to a nucleotide region
comprising a DNA regulatory sequence, wherein the
regulatory sequence is derived from a gene which is
capable of binding RNA polymerase and initiating
transcription of a downstream (3'-direction) coding

25 sequence. Certain consensus sequences within the
promoter region are deemed to be particularly
important in the binding of RNA polymerase, and are
generally referred to as CAT and TATA boxes. Promoter
regions extend from about 40 nucleotides to about 5

30 nucleotides upstream from the start of the gene-coding
region, the CAT and TATA boxes being located within
the promoter region as short stretches of nucleotide
sequences. The TATA box includes the binding site of
transcription factors, but not of the RNA polymerase

35 enzyme.

An "AAV p5 promoter region" encompasses both
promoter sequences with identity to a p5 promoter

region isolated from an AAV serotype, including
without limitation, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5,
AAVX7, etc., as well as those which are substantially
homologous and functionally equivalent thereto (as

5   defined below). The AAV p5 promoter directs the
expression of the long forms of Rep, and has been
described and characterized. *See, e.g.*, Lusby et al.
(1982) *J. Virol.* 41:518-526; Laughlin et al. (1979)
Proc. Natl. Acad. Sci. USA 76:5567-5571; Green et al.

10   (1980a) *J. Virol.* 36:79-92; Green et al. (1980b) *Cell*
1:231-242. For purposes of defining the present
invention, in the wt AAV genome, the AAV p5 promoter
region is "in its natural position" when it is bound
at the 5'-terminus of the transcriptional start site

15   of the *rep* coding sequence and the *rep* transcriptional
start site is approximately 25 bps downstream (3'-
direction) from the p5 TATA box, such that the *rep* ATG
is approximately 60 bps downstream (3'-direction) from
the p5 TATA box. The wt AAV p5 promoter extends

20   upstream (5'-direction) to include the minimum number
of bases or elements necessary to initiate
transcription of the long forms of Rep at levels
detectable above background.

       An AAV p5 promoter region is situated "other
25   than in its natural position" when the AAV p5 promoter
has been moved from its natural position relative to
the *rep* coding sequence in the particular nucleic acid
molecule being described. For example, an AAV p5
promoter region "is situated other than in its natural

30   position" when that region is situated in a nucleic
acid molecule such that the AAV p5 promoter TATA box
is not 25 bps upstream (5'-direction) of the *rep*
transcriptional start site in that same molecule.

       By "AAV *rep* coding region" is meant the art-
35   recognized region of the AAV genome which encodes the
replication proteins of the virus which are
collectively required for replicating the viral

genome, or functional homologues thereof such as the
human herpesvirus 6 (HHV-6) *rep* gene which is also
known to mediate AAV-2 DNA replication (Thomson et al.
(1994) *Virology* 204:304-311). Thus, the *rep* coding

5   region includes at least the genes encoding for AAV
Rep 78, Rep 68, Rep 52 and Rep 40, or functional
homologues thereof. As used herein, the *rep* coding
region does not include the AAV p5 promoter region.
For a further description of the AAV *rep* coding

10   region, *see, e.g.,* Muzyczka, N. (1992) *Current Topics
in Microbiol. and Immunol.* 158:97-129; and Kotin, R.M.
(1994) *Human Gene Therapy* 5:793-801. The *rep* coding
region can be derived from any serotype, such as those
AAV serotypes described above. The region need not

15   include all of the wild-type genes but may be altered,
e.g., by the insertion, deletion or substitution of
nucleotides, so long as the *rep* genes present provide
for sufficient replication functions when present in a
host cell along with an AAV vector.

20   The term "short forms of Rep" refers to the
Rep 52 and Rep 40 gene products of the AAV *rep* coding
region, including functional homologues thereof. The
short forms of Rep are expressed under the direction
of the AAV p19 promoter which has been described and

25   characterized. *See e.g.,* Lusby et al., Laughlin et
al., Green et al. (1980a) and Green et al. (1980b),
supra.
           The term "long forms of Rep" refers to the
Rep 78 and Rep 68 gene products of the AAV *rep* coding

30   region, including functional homologues thereof. The
long forms of Rep are normally expressed under the
direction of the AAV p5 promoter which has been
described and characterized. *See,* Lusby et al.,
Laughlin et al., Green et al. (1980a) and Green et al.

35   (1980b), *supra.*
           By "AAV *cap* coding region" is meant the art-
recognized region of the AAV genome which encodes the

coat proteins of the virus which are collectively
required for packaging the viral genome. Thus, the
cap coding region includes at least the genes encoding
for the coat proteins VP1, VP2 and VP3. For a further
5   description of the *cap* coding region, *see, e.g.*,
Muzyczka, N. (1992) *Current Topics in Microbiol. and
Immunol.* 158:97-129; and Kotin, R.M. (1994) *Human Gene
Therapy* 5:793-801. The AAV *cap* coding region, as used
herein, can be derived from any AAV serotype, as
10   described above. The region need not include all of
the wild-type *cap* genes but may be altered, e.g., by
the insertion, deletion or substitution of
nucleotides, so long as the genes provide for
sufficient packaging functions when present in a host
15   cell along with an AAV vector.

By an "AAV coding region" is meant a nucleic
acid molecule which includes the two major AAV open
reading frames corresponding to the AAV *rep* and *cap*
coding regions (e.g., a nucleic acid molecule
20   comprising a nucleotide sequence substantially
homologous to base pairs 310 through 4,440 of the
wild-type AAV genome). *See, e.g.*, Srivastava et al.
(1983) *J. Virol.* 45:555-564; Hermonat et al. (1984) *J.
Virol.* 51:329-339; and Tratschin et al. (1984) *J.
25   Virol.* 51:611-619. Thus, for purposes of the present
invention, an "AAV coding region" does not include
those sequences corresponding to the AAV p5 promoter
region, and does not include the AAV ITRs.

A "Flip Recombination Target site" (FRT)
30   refers to a nucleotide sequence that serves as a
substrate in the site-specific yeast flip recombinase
system. The FRT recombination region has been mapped
to an approximately 65-base pair (bp) segment within
the 599-bp long inverted repeats of the 2-μm circle (a
35   commonly occurring plasmid in *Saccharomyces
cerevisiae*). The enzyme responsible for recombination
(FLP) is encoded by the 2-μm circle, and has been

expressed at high levels in human cells. FLP
catalyzes recombination within the inverted repeats of
the molecule to cause intramolecular inversion. FLP
can also promote efficient recombination between

5 plasmids containing the 2-μm circle repeat with very
high efficiency and specificity. *See, e.g.*, Jayaram
(1985) *Proc. Natl. Acad. Sci. USA* 82:5875-5879; and
O'Gorman (1991) *Science* 251:1351-1355. A "minimum FRT
site" (e.g., a minimal FLP substrate) has been

10 described in the art and is defined herein as a 13-bp
dyad symmetry plus an 8-bp core located within the 65-
bp FRT region. Jayaram et al., *supra*. Both FRT sites
and FLP expression plasmids are commercially available
from Stratagene (San, Diego, CA).

15 "Transfection" refers to the uptake of
foreign DNA by a cell, and a cell has been
"transfected" when exogenous DNA has been introduced
inside the cell membrane. In this manner, the
exogenous DNA may or may not become integrated

20 (covalently linked) to chromosomal DNA making up the
genome of the cell. A number of transfection
techniques are known in the art. *See, e.g.*, Graham et
al. (1973) *Virology*, 52:456, Sambrook et al. (1989)
Molecular Cloning, a laboratory manual, Cold Spring

25 Harbor Laboratories, New York, Davis et al. (1986)
Basic Methods in Molecular Biology, Elsevier, and Chu
et al. (1981) *Gene* 13:197. Any of these techniques
can be used to introduce one or more exogenous DNA
moieties, such as AAV helper constructs, AAV vector

30 plasmids, and other vector constructs, into suitable
host cells. Generally, the exogenous DNA must
traverse the recipient cell plasma membrane in order
to be exposed to the cell's transcription and
replication machinery. The resulting cell can either

35 be transiently transfected with the exogenous nucleic
acid molecule, or stably transfected--wherein the
nucleic acid molecule is covalently linked with the

host cell genome or maintained and replicated as an
episomal unit which can be passed on to progeny cells
(e.g., capable of extra-chromosomal replication at a
sufficient rate). Such transfection methods have been
5   described in the art, including calcium phosphate co-
precipitation (Graham et al. (1973) *Virol.* 52:456-
467), direct micro-injection into cultured cells
(Capecchi, M.R. (1980) *Cell* 22:479-488),
electroporation (Shigekawa et al. (1988) *BioTechniques*
10   6:742-751), liposome mediated gene transfer (Mannino
et al. (1988) *BioTechniques* 6:682-690), lipid-mediated
transfection (Felgner et al. (1987) *Proc. Natl. Acad.
Sci. USA* 84:7413-7417), and nucleic acid delivery
using high-velocity microprojectiles (Klein et al.
15   (1987) *Nature* 327:70-73).

"Transient transfection" refers to cases
where exogenous DNA does not integrate into the genome
of a transfected cell, e.g., where episomal DNA is
transcribed into mRNA and translated into protein.
20   A cell has been "stably transfected" with a
nucleic acid construct comprising AAV coding regions
when the nucleic acid construct has been introduced
inside the cell membrane and the AAV coding regions
are capable of being inherited by daughter cells.
25   The term "host cell" denotes, for example,
microorganisms, yeast cells, insect cells, and
mammalian cells, that can be, or have been, used as
recipients of an AAV helper construct, an AAV vector
plasmid, or other transfer DNA, and include the
30   progeny of the original cell which has been
transfected. Thus, a "host cell" as used herein
generally refers to a cell which has been transfected
with an exogenous DNA sequence using methods as
described above. It is understood that the progeny of
35   a single parental cell may not necessarily be
completely identical in morphology or in genomic or

total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

As used herein, the term "cell line" refers to a population of cells capable of continuous or
5   prolonged growth and division *in vitro*. Often, cell lines are clonal populations derived from a single progenitor cell. It is further known in the art that spontaneous or induced changes can occur in karyotype during storage or transfer of such clonal populations.
10   Therefore, cells derived from the cell line referred to may not be precisely identical to the ancestral cells or cultures, and the cell line referred to includes such variants.

A "packaging cell" refers to a host cell
15   which, by way of stable or transient transfection with heterologous nucleotide sequences, harbors a nucleic acid molecule comprising an AAV helper construct, wherein the construct is capable of providing transient expression of AAV helper functions that can
20   be provided in *trans* for productive AAV replication. Expression of the AAV helper functions can be either constitutive, or inducible, such as when the helper functions are under the control of an inducible promoter.
25   A "producer cell" refers to a packaging cell that has been stably or transiently transfected with an AAV vector--either before, subsequent to, or at the same time as transfection of the cell with the AAV helper functions. In this manner, a producer cell
30   contains AAV sequences that are provided in *cis* for replication and packaging (e.g., functional ITR sequences), and AAV sequences encoding helper functions missing from the AAV vector and provided in *trans* for replication and packaging. In the presence
35   of requisite viral helper functions, the producer cell is thus capable of encoding AAV polypeptides that are required for packaging transfected viral DNA (e.g.,

AAV viral vectors containing a recombinant nucleotide
sequence of interest) into infectious viral particles
for subsequent gene delivery.

Viral helper functions, as defined above,
5   can be introduced into a producer cell by infection or
superinfection thereof with one or more helper virus
moiety such as an adenovirus, herpesvirus or vaccinia
virus.

The term "heterologous" as it relates to
10 nucleic acid sequences such as coding sequences and
control sequences, denotes sequences that are not
normally joined together, and/or are not normally
associated with a particular cell. Thus, a
"heterologous" region of a nucleic acid construct is a
15 segment of nucleic acid within or attached to another
nucleic acid molecule that is not found in association
with the other molecule in nature. For example, a
heterologous region of a construct could include a
coding sequence flanked by sequences not found in
20 association with the coding sequence in nature.
Another example of a heterologous coding sequence is a
construct where the coding sequence itself is not
found in nature (e.g., synthetic sequences having
codons different from the native gene). Similarly, a
25 host cell transformed with a construct which is not
normally present in the cell would be considered
heterologous for purposes of this invention. Allelic
variation or naturally occurring mutational events do
not give rise to heterologous DNA, as used herein.
30   A "coding sequence" or a sequence which
"encodes" a particular polypeptide, is a nucleic acid
sequence which is transcribed (in the case of DNA) and
translated (in the case of mRNA) into a polypeptide *in
vitro* or *in vivo* when placed under the control of
35 appropriate regulatory sequences. The boundaries of
the coding sequence are determined by a start codon at
the 5' (amino) terminus and a translation stop codon

at the 3' (carboxy) terminus. A coding sequence can
include, but is not limited to, cDNA from procaryotic
or eukaryotic mRNA, genomic DNA sequences from
procaryotic or eukaryotic DNA, and even synthetic DNA

5   sequences. A transcription termination sequence will
usually be located 3' to the coding sequence.

        The term DNA "control sequences" refers
collectively to promoter sequences, polyadenylation
signals, transcription termination sequences, upstream

10   regulatory domains, origins of replication, internal
ribosome entry sites ("IRES"), enhancers, and the
like, which collectively provide for the replication,
transcription and translation of a coding sequence in
a recipient cell. Not all of these control sequences

15   need always be present so long as the selected gene is
capable of being replicated, transcribed and
translated in an appropriate recipient cell.

        "Operably linked" refers to an arrangement
of elements wherein the components so described are

20   configured so as to perform their usual function.
Thus, control sequences operably linked to a coding
sequence are capable of effecting the expression of
the coding sequence. The control sequences need not
be contiguous with the coding sequence, so long as

25   they function to direct the expression thereof. Thus,
for example, intervening untranslated yet transcribed
sequences can be present between a promoter sequence
and the coding sequence and the promoter sequence can
still be considered "operably linked" to the coding

30   sequence.

        By "isolated" when referring to a nucleotide
sequence, is meant that the indicated molecule is
present in the substantial absence of other biological
macromolecules of the same type. Thus, an "isolated

35   nucleic acid molecule which encodes a particular
polypeptide" refers to a nucleic acid molecule which
is substantially free of other nucleic acid molecules

that do not encode the subject polypeptide; however,
the molecule may include some additional bases or
moieties which do not deleteriously affect the basic
characteristics of the composition.

5   For the purpose of describing the relative
position of nucleotide sequences in a particular
nucleic acid molecule throughout the instant
application, such as when a particular nucleotide
sequence is described as being situated "upstream,"
10 "downstream," "3'," or "5'" relative to another
sequence, it is to be understood that it is the
position of the sequences in the "sense" or "coding"
strand of a DNA molecule that is being referred to as
is conventional in the art.

15   "Homology" refers to the percent of identity
between two polynucleotide or two polypeptide
moieties. The correspondence between the sequence
from one moiety to another can be determined by
techniques known in the art. For example, homology
20 can be determined by a direct comparison of the
sequence information between two polypeptide molecules
by aligning the sequence information and using readily
available computer programs. Alternatively, homology
can be determined by hybridization of polynucleotides
25 under conditions which form stable duplexes between
homologous regions, followed by digestion with single-
stranded-specific nuclease(s), and size determination
of the digested fragments. Two DNA, or two
polypeptide sequences are "substantially homologous"
30 to each other when at least about 80%, preferably at
least about 90%, and most preferably at least about
95% of the nucleotides or amino acids match over a
defined length of the molecules, as determined using
the methods above.

35   A "functional homologue," or a "functional
equivalent" of a given polypeptide includes molecules
derived from the native polypeptide sequence, as well

as recombinantly prod...ed or chemically synthesized
polypeptides which function in a manner similar to the
reference molecule to achieve a desired result. Thus,
a functional homologue of AAV Rep 52 or Rep 40

5   encompasses derivatives and analogues of those
polypeptides--including any single or multiple amino
acid additions, substitutions and/or deletions
occurring internally or at the amino or carboxy
termini thereof, so long as replication activity

10   remains.

A "functional homologue," or a "functional
equivalent" of a given AAV promoter region includes
promoters derived from an AAV serotype, as well as
recombinantly produced or chemically synthesized

15   polynucleotides which function in a manner similar to
the reference promoter region to achieve a desired
result. Thus, a functional homologue of an AAV p5
promoter region encompasses derivatives and analogues
of such control sequences--including any single or

20   multiple nucleotide base additions, substitutions
and/or deletions occurring within the promoter region,
so long as the promoter homologue retains the minimum
number of bases or elements sufficient to initiate
transcription of the long forms of Rep at levels

25   detectable above background.

B. General Methods
It is a primary object of the invention to
provide improved AAV helper systems useful in the

30   production of recombinant AAV (rAAV) virions that can
subsequently be used in gene transfer methods.
Particularly, it is an object of the invention to
develop AAV helper constructs that can be introduced
into suitable packaging cells to provide for enhanced

35   production of commercially useful levels of
recombinant AAV virions.

In one particular embodiment, a nucleic acid molecule is provided having an AAV *rep* and *cap* coding region and an AAV p5 promoter region that is situated in the subject molecule at a site that is other than
5 its normal position relative to the AAV *rep* coding region in a wild-type (wt) AAV genome. The *rep* and *cap* coding regions can be arranged in the molecule as two contiguous main open reading frames, respectively arranged in the order given in the 5' to 3' direction
10 such as the normal arrangement of those coding regions in the wt AAV genome. *See, e.g.,* Berns and Bohenzky (1987) *Advances in Virus Research* (Academic Press, Inc.) 32:243-307; Tratschin et al. (1984) *J. Virol.* 51:611-619; and Srivastava et al. (1983) *J. Virol.*
15 45:555-564.
    Alternatively, the *rep* and *cap* coding regions can be arranged in the nucleic acid molecule as two non-contiguous regions separated by intervening nucleotides and arranged in any order--so long as the
20 *rep* coding region includes at least one promoter capable of directing the expression of *rep52/40* (such as a nucleotide sequence that is substantially homologous to an AAV p19 promoter region), and the *cap* coding region includes at least one promoter capable
25 of directing the expression of the Cap expression products (such as a nucleotide sequence that is substantially homologous to an AAV p40 promoter region).
    The instant molecules can be constructed by
30 linking nucleotide sequences corresponding to AAV *rep* and *cap* coding regions (Srivastava et al., *supra.*) with a nucleotide sequence that is substantially homologous to an AAV p5 promoter region, or that corresponds to bps 145-309 of the wt AAV genome
35 (Figure 4, [SEQ ID NO:4])--wherein the p5 promoter region is arranged in the molecule in any position other than its normal position relative to the *rep*

coding region. In one particular molecule, the
nucleotide sequence that comprises the AAV p5 promoter
region is substantially homologous to bps 145-494 of
the wt AAV genome (Figure 4, [SEQ ID NO:4]). In

5   another molecule, the nucleotide sequence that is
homologous to the AAV p5 promoter region is positioned
downstream from both the *rep* and *cap* coding regions,
substantially adjacent to the 3' terminus of the *cap*
coding region. By substantially adjacent to the 3'

10 terminus is meant that the subject nucleotide sequence
is within about 0 to 500 nucleotides, more preferably
within about 0 to 200 nucleotides, and most preferably
within about 0 to 50 nucleotides of the 3' terminus of
the *cap* coding region.

15   In yet another embodiment, the AAV p5
promoter region is positioned downstream from both the
rep and *cap* coding regions and separated therefrom by
an intervening nucleotide sequence (X) having a
minimum length (ℓ), whereby the resultant AAV *rep-cap-*

20 *X-p5* fragment is sized such that recombination events
(during recombinant AAV virion production) between
said fragment and an AAV vector (resulting in an
acquisition of ITRs) will yield a recombined molecule
that is too large to package as an AAV virion. It is

25 generally recognized that an approximately 5100 bp
fragment represents the upper limit of genetic
material which can be packaged in such particles (*see,*
e.g., U.S. Patent No. 5,173,414 to Lebkowski et al.).
In this way, the potential production of contaminating

30 wild-type AAV particles is reduced or eliminated. In
one particular molecule, the intervening nucleotide
sequence (X) comprises at least 500 base pairs.
       The above-described nucleic acid molecules
can be isolated and cloned into a suitable vector such

35 as a plasmid or virus particle to provide an AAV
helper construct, wherein the vector can further
include suitable control elements for replication and

expression of the AAV coding sequences and facilitates
the transfer of the nucleic acid molecule between
cells.

                In additional embodiments of the invention,

5   the above-described nucleic acid molecules can include
one or more nucleotide sequences that are
substantially homologous to yeast FLP recombinase
substrates (e.g. Flip Recombination Target (FRT)
sites). Jayaram (1985) *Proc. Natl. Acad. Sci. USA*

10   82:5875-5879; and O'Gorman (1991) *Science* 251:1351-
1355. Thus, the above-described nucleic acid
molecules can include a second nucleotide sequence
that is homologous to an FRT site (or at least a
minimum FRT site), wherein the second nucleotide

15   sequence is arranged in the molecule such that the FRT
site is situated upstream of the AAV coding regions
and the AAV p5 promoter region. In preferred
embodiments, molecules are provided which comprise, in
the 5' to 3' direction, an FRT site (Jayaram, and

20   O'Gorman, *supra*), an AAV *rep* coding region, an AAV *cap*
coding region, and an AAV p5 promoter region. In yet
a further embodiment, a molecule is provided wherein
the nucleotide sequence that contains the AAV p5
promoter region is substantially homologous to bps

25   145-494 of the wt AAV genome (Figure 4 [SEQ ID NO:4]).
This first nucleotide sequence also has an inserted
polynucleotide that comprises an FRT site. More
particularly, a polynucleotide insert (comprising an
FRT site) has been placed between bps 310 and 311 of

30   the first sequence so that the inserted FRT site is
situated immediately adjacent the 3' terminus of the
AAV p5 promoter region (which extends from bps 145-
310). The resultant construct is then positioned in
the nucleic acid molecule to situate the AAV p5

35   promoter region in any position other than its normal
position relative to the *rep* coding region, as has
been described above. All of the aforementioned

molecules can be constructed using recombinant techniques known in the art.

In related embodiments, the above-described nucleic acid molecules are constructed so as to include a plurality of polynucleotides that are homologous to an FRT site. More particularly, two FRT sites can be arranged in the molecules so that they provide 5' and 3' flanking regions bordering the AAV coding regions and AAV p5 promoter region. In this manner, the nucleic acid molecule comprises a cassette (having an AAV *rep* coding region, an AAV *cap* coding region and an AAV p5 promoter region) that is flanked by FRT sites. In preferred embodiments, the nucleic acid molecules are arranged such that they comprise, in the order given in the 5' to 3' direction, an FRT site, AAV *rep*, AAV *cap*, an AAV p5 promoter region, and an FRT site. The above molecules can be assembled, isolated and cloned into a suitable vector using known techniques. The FRT-*rep*-*cap*-p5-FRT cassette can be readily inserted into, or excised from a vector by the action of the yeast FLP recombinase enzyme if so desired.

In yet a further related embodiment, a molecule is provided having AAV *rep* and *cap* coding regions, a nucleotide sequence containing an AAV p5 promoter region, and a plurality of FRT sites. In this particular molecule, the nucleotide sequence that contains the AAV p5 promoter region is substantially homologous to bps 145-494 of the wt AAV genome (Figure 4 [SEQ ID NO:4]). Within this first nucleotide sequence, there has been inserted a polynucleotide that comprises an FRT site. More particularly, a polynucleotide insert (comprising an FRT site) has been placed between bps 310 and 311 of the first sequence so that the inserted FRT site is situated immediately adjacent the 3' terminus of the AAV p5 promoter region (which extends from bps 145-310). The

-28-

nucleic acid molecule is then arranged such that it comprises, in the order given in the 5' to 3' direction, an FRT site, AAV *rep*, AAV *cap*, and a nucleotide sequence comprising an AAV p5 promoter region and having an FRT site situated immediately 3' of the AAV p5 promoter region.

Furthermore, vectors containing the above-described nucleic acid molecules are readily introduced into a suitable host and expressed therein to complement missing AAV functions in AAV vectors that lack functioning *rep* and/or *cap* coding regions. The *rep* and *cap* regions in an AAV vector can be disabled by deletions of genetic material, insertions of genetic material that cause reading frame errors and point mutations that disrupt the replication and encapsidation functions supplied by those genes. An AAV vector system can be screened for a functioning *rep* coding region by transfecting the vector into a suitable host, such as an adenovirus-infected cell, and assaying cell extracts, e.g., 48 hours later, for the presence of replicating vector genomes. If the *rep* coding region is functional, replicating DNA can be revealed by Southern blot analysis using techniques known in the art. AAV vector systems can be screened for a functioning *cap* coding region by assaying for AAV particle production using Western blot techniques that are known in the art (Samulski et al. (1989) *J. Virol.* 63:3822-3828).

The nucleic acid molecules of the present invention can be constructed using conventional recombinant techniques. In this regard, nucleic acid molecules containing AAV *rep* and *cap* coding regions with a displaced AAV p5 promoter region can be readily constructed by inserting a nucleotide sequence that includes an AAV p5 promoter region into a construct having an AAV coding region (containing *rep* and *cap* coding regions) by ligating a restriction fragment

containing the subject promoter region into a suitable
site relative to the AAV *rep* coding region. The newly
formed nucleic acid molecule can then be excised from
the construct using restriction enzymes if so desired.
5   These and other molecules of the invention can thus be
provided herein using techniques well known in the
art. *See, e.g.*, U.S. Patent Nos. 5,173,414 and
5,139,941; International Publication Nos. WO 92/01070
(published 23 January 1992) and WO 93/03769 (published
10   4 March 1993); Lebkowski et al. (1988) *Molec. Cell.
Biol.* 8:3988-3996; Vincent et al. (1990) *Vaccines 90*
(Cold Spring Harbor Laboratory Press); Carter, B.J.
(1992) *Current Opinion in Biotechnology* 3:533-539;
Muzyczka, N. (1992) *Current Topics in Microbiol. and
15   Immunol.* 158:97-129; Kotin, R.M. (1994) *Human Gene
Therapy* 5:793-801; Shelling and Smith (1994) *Gene
Therapy* 1:165-169; and Zhou et al. (1994) *J. Exp. Med.*
179:1867-1875.
          More particularly, selected AAV coding
20   regions comprising the *rep* and *cap* genes, and selected
nucleotide sequences such as those containing an AAV
p5 promoter region, can be excised from the viral
genome or from an AAV vector containing the same and
linked such that the p5 promoter region is 3' of the
25   *rep* and/or *cap* coding regions, using standard ligation
techniques such as those described in Sambrook et al.,
supra. The molecules can be further constructed to
have flanking FRT sequences arranged at their 5', or
5' and 3' ends. Ligations can be accomplished in 20
30   mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 33 ug/ml
BSA, 10 mM-50 mM NaCl, and either 40 uM ATP, 0.01-0.02
(Weiss) units T4 DNA ligase at 0°C (for "sticky end"
ligation) or 1 mM ATP, 0.3-0.6 (weiss) units T4 DNA
ligase at 14°C (for "blunt end" ligation).
35   Intermolecular "sticky end" ligations are usually
performed at 30-100 µg/ml total DNA concentrations
(5-100 nM total end concentration).

-31-

              In the alternative, the nucleic acid
      molecules of the invention can be synthetically
      derived, using a combination of solid phase direct
      oligonucleotide synthesis chemistry and enzymatic
5   ligation methods which are conventional in the art.
      Synthetic sequences may be constructed having features
      such as restriction enzyme sites and can be prepared
      in commercially available oligonucleotide synthesis
      devices such as those devices available from Applied
10   Biosystems, Inc. (Foster City, CA) using the
      phospharamidite method. See, e.g., Beaucage et al.
      (1981) *Tetrahedron Lett.* 22:1859-1862. The nucleotide
      sequences of the AAV *rep* and *cap* coding sequences, the
      AAV p5, p19 and p40 promoter regions, and FRT sites
15   are known and have been previously described, (*see,
      e.g.,* Srivastava et al. (1983) *J. Virol.* 45:555-564;
      Kotin, R.M. (1994) *Human Gene Therapy* 5:793-801;
      Berns, K.I. "Parvoviridae and their Replication" in
      Fundamental Virology, 2nd Edition, (B.N. Fields and
20   D.M. Knipe, eds.) for AAV sequences; and, *see, e.g.,*
      Jayaram et al. (1985) *Proc. Natl. Acad. Sci. USA*
      82:5875-5879 and O'Gorman (1991) *Science* 251:1351-1355
      for FRT sequences). Preferred codons for expression
      of the synthetic molecule in mammalian cells can also
25   be synthesized. Complete nucleic acid molecules are
      then assembled from overlapping oligonucleotides
      prepared by the above methods. *See, e.g.,* Edge,
      Nature (1981) 292:756; Nambair et al. *Science* (1984)
      223:1299; Jay et al. *J. Biol. Chem.* (1984) 259:6311.
30   It is a further object of the invention to
      provide AAV helper constructs that generally comprise
      replicons including the nucleic acid molecules of the
      present invention. The constructs are thus capable of
      encoding AAV helper functions. A replicon is defined
35   herein as any length of DNA that serves as a unit of
      replication during DNA synthesis. In one embodiment,
      an AAV helper construct is provided in the form of an

episome that is capable of autonomous replication
independently of a host genome, and which may further
be capable of integration into a host chromosome. In
one particularly preferred embodiment, the construct

5   is a plasmid. In various other embodiments, the AAV
helper functions (e.g., the *rep* and *cap* coding
regions) are operably linked to control sequences that
direct the transcription and translation thereof.

       In one particular aspect of the invention,

10 AAV helper constructs are assembled so as to provide
expression cassettes that can be maintained as an
extrachromosomal replicon (e.g., an episome or
plasmid) that is capable of stable maintenance in a
host cell. The construct will have an appropriate

15 replication system allowing it to be substantially
stably maintained in a replicating host.

       AAV helper constructs which include the AAV
rep and *cap* coding regions with an AAV p5 promoter
region--arranged in the subject construct to be

20 situated in a site other than its normal position
relative to the *rep* coding region in the wt AAV
genome--control sequences and optional amplification
sequences, as described above, can also include
selectable markers. Suitable markers include genes

25 which confer antibiotic resistance or sensitivity, or
impart color, or change the antigenic characteristics
when cells which have been transfected with the
nucleic acid constructs are grown in an appropriate
selective medium. Particular selectable marker genes

30 useful in the practice of the invention include the
hygromycin B resistance gene (encoding Aminoglycoside
phosphotranferase (APH)) that allows selection in
mammalian cells by conferring resistance to G418
(available from Sigma, St. Louis, Mo.). Other

35 suitable markers are known to those of skill in the
art.

It is yet a further object of the invention to provide AAV packaging cells that are capable of producing rAAV virions when an AAV vector is present in the cell and the packaging cell is capable of

5   expressing viral helper functions. In one particular embodiment, AAV packaging cells can be derived from mammalian cells which are able to sustain infection by a helper virus (for the provision of viral helper functions). In this regard, almost any mammalian cell

10   can sustain AAV and produce AAV virions so long as a helper virus is present which is compatible with the cell. Therefore, packaging cells used to produce rAAV virions will be a matter of choice, largely dictated by convenience, such as availability, growth

15   characteristics, or the like. Suitable packaging cells include, without limitation, cells derived from human and nonhuman primate species, rodent, bovine, ovine, porcine, equine, feline and canine cells, among others. However, due to convenience, human cell lines

20   are preferred such as human 293, HeLa, KB and JW-2 cells. These cells are readily available through the American Type Culture Collection (ATCC) (e.g., human 293 cells are available under accession number ATCC CRL1573).

25   In one particular embodiment, an AAV packaging cell is formed from a suitable host cell (e.g., a human 293 cell) by transfecting the cell with an AAV helper construct capable of expressing AAV helper functions. The subject AAV helper construct

30   comprises a nucleic acid molecule having AAV *rep* and *cap* coding regions and a nucleotide sequence comprising an AAV p5 promoter region, wherein the nucleotide sequences are arranged in the molecule so as to situate the p5 promoter region in a site that is

35   other than its normal position relative the *rep* coding region in the wt AAV genome as has been described above. The helper construct is capable of being

efficiently transcribed and translated in the host
cell to complement missing AAV functions in an
associated AAV vector.

In another particular embodiment, an AAV
packaging cell is formed from a suitable host cell
(e.g., a human 293 cell) by transfection with an AAV
helper construct capable of expressing AAV Rep and Cap
polypeptides. The transfected AAV helper construct
comprises a nucleic acid molecule having an AAV coding
region and a nucleotide sequence comprising an AAV p5
promoter region, said AAV coding region and said p5
promoter region respectively arranged 5' to 3' in the
molecule and separated from each other by an
intervening nucleotide sequence. The subject
construct is capable of being efficiently transcribed
and translated in the host cell to complement missing
AAV functions in an associated AAV vector. Further,
the intervening nucleotide sequence can be selected to
have a sufficient length that renders the resultant
AAV *rep-cap...p5* fragment too large to package as an
AAV virion particle in the event of recombination
during rAAV virion production. In this manner,
production of significant levels of contaminating wt
AAV particles in the packaging cell system is avoided.
In a related embodiment, the AAV helper construct
comprises a plasmid.

Each of the AAV helper constructs of the
invention can be stably maintained in the packaging
cell as an episomal element or can be integrated into
the packaging cell genome, thus creating a packaging
cell line which can be maintained indefinitely.
Alternatively, the AAV helper constructs can be
transfected into the packaging cell either just prior
or subsequent to, or concomitant with, introduction of
suitable viral helper functions.

It is also an object of the present
invention to provide AAV producer cells that are

capable of producing rAAV virions when viral helper
functions are expressed therein. In one particular
embodiment, producer cells are formed by either
transiently or stably transfecting one of the

5   aforementioned AAV packaging cells with an AAV vector
(such as a plasmid) harboring a heterologous
nucleotide sequence that is interposed between
functional AAV ITRs.

It is yet an even further object of the

10   invention to provide methods for the production of
rAAV virions, wherein the methods generally involve
the steps of (1) introducing an AAV vector harboring a
heterologous nucleotide sequence to be transduced that
is interposed between functional AAV ITRs into a host

15   cell; (2) introducing an AAV helper construct that has
been assembled as described above into the host cell,
wherein the construct is capable of expressing AAV
helper functions missing from the AAV vector; (3)
expressing viral helper functions in the host cell;

20   and (4) culturing the cell to produce rAAV virions.

In one embodiment, a method of producing
rAAV virions is provided wherein an AAV packaging cell
that has been constructed as described above is
transfected with an AAV vector containing a

25   heterologous nucleotide sequence of interest that is
interposed between AAV ITRs. The AAV packaging cell
can be either transiently or stably transfected with
the subject AAV vector (to provide a producer cell as
has been described above).

30   In one preferred embodiment, the AAV
packaging cell used in the above method is produced by
transfecting a suitable host cell with an AAV helper
construct capable of being expressed to provide AAV
Rep and Cap polypeptides. The subject AAV helper

35   construct comprises a nucleic acid molecule having AAV
rep and *cap* coding regions and a nucleotide sequence
comprising an AAV p5 promoter region, wherein the

elements of the molecule are arranged so as to situate
the p5 promoter region in a site that is other than
its normal position relative the *rep* coding region in
the wt AAV genome as has been described above.   The

5   AAV helper construct can be co-transfected into the
host cell with an appropriate AAV vector using methods
known to those skilled in the art.   In further related
embodiments, the AAV helper construct can include one
or more flanking FRT sites as described *supra*.

10   In another preferred embodiment, an AAV
packaging cell is produced by transfecting a suitable
host cell with an AAV helper construct designed to
reduce or eliminate the production of significant
levels of wild-type AAV during rAAV vector production.

15   The helper construct includes a nucleic acid molecule
having AAV *rep* and *cap* coding regions, an intervening
nucleotide sequence, and a nucleotide sequence
comprising an AAV p5 promoter region.   The elements of
the molecule are arranged such that the AAV p5

20   promoter region is positioned downstream from both the
rep and cap coding regions and separated therefrom by
the intervening nucleotide sequence (X).   The
nucleotide sequence (X) is selected to have a minimum
length such that the resultant AAV *rep-cap*-X-p5

25   fragment will have an overall size that is effective
to ensure that recombination events (during
recombinant AAV virion production) between said
fragment and an AAV vector (resulting in an
acquisition of ITRs) will yield a recombined molecule

30   that is too large to package as an AAV virion.   In
this manner, the potential production of wt AAV
particles is reduced or eliminated.

                In the practice of the invention, enhanced
titers of rAAV virions can be obtained using methods

35   which employ the above-described packaging cells.   Not
being bound by any particular theory, enhanced virion
production in such cells is thought to be due in part

to the attenuation of Rep toxicity in those cells. In
this regard, the placement of the p5 promoter region
in a site that is other than its normal position
relative to the *rep* coding region may serve to
5   attenuate production of both Rep 78 and Rep 68 (the
long form Rep expression products normally transcribed
from the p5 promoter) when the *rep* coding region is
expressed. In light of the fact that some long form
Rep products are expressed from the present AAV helper
10   constructs, the relocated AAV p5 promoter region may
serve an effector function by virtue of its new
position.
        In each of the above-described methods,
viral helper functions can be expressed in the host
15   cells using methods that are known to those of skill
in the art. Particularly, viral helper functions are
provided by infection of the host cells with an
unrelated helper virus. Helper viruses which will
find use with the present systems include the
20   adenoviruses; herpesviruses such as herpes simplex
virus types 1 and 2; and vaccinia viruses. Nonviral
helpers will also find use herein, such as cell
synchronization, using any of various known agents.
See, e.g., Buller et al. (1981) *J. Virol.* 40:241-247;
25   McPherson et al. (1985) *Virology* 147:217-222;
Schlehofer et al. (1986) *Virology* 152:110-117. As a
consequence of the infection of the host cell, the
viral helper functions are capable of being expressed
to transactivate an AAV helper construct to produce
30   AAV Rep and Cap proteins. In this manner, the Rep
proteins serve to excise the recombinant DNA
(containing the heterologous nucleotide sequence) from
the recombinant AAV vector (or from the host cell
genome if the AAV vector has been integrated). The
35   Rep proteins also serve to duplicate the AAV genome.
The expressed Cap proteins assemble into capsids, and
the recombinant AAV genome is packaged into the

capsids. Thus, lytic AAV replication ensues, and the
heterologous nucleotide sequence is packaged into
viable transducing vectors.

Following expression of the viral helper
5   functions in the host cell and the AAV replication,
rAAV virions can be purified from the host cell using
a variety of conventional purification methods, such
as CsCl gradients. Further, if infection is employed
to express the viral helper functions, any residual
10   helper virus can be inactivated, using known methods.
For example, adenovirus can be inactivated by heating
to temperatures of approximately 60°C for, e.g., 20
minutes or more, since AAV is extremely heat stable
and adenovirus is heat labile.

15   The resulting rAAV virions containing the
heterologous nucleotide sequence of interest can then
be used for gene delivery, such as in gene therapy
applications, for the production of transgenic
animals, in vaccination, ribozyme and antisense
20   therapy, as well as for the delivery of genes *in
vitro*, to a variety of cell types.

C.   Experimental

Below are examples of specific embodiments
25   for carrying out the present invention. The examples
are offered for illustrative purposes only, and are
not intended to limit the scope of the present
invention in any way.

Efforts have been made to ensure accuracy
30   with respect to numbers used (*e.g.*, amounts,
temperatures, etc.), but some experimental error and
deviation should, of course, be allowed for.

Example 1

35   Construction of the pAAVlacZ Plasmid

An AAV vector carrying the *lacZ* gene
(pAAV-lacZ) was constructed as follows. The AAV

-38-

coding region of pSub201 (Samulski et al. (1987) *J.*
Virol 61:3096-3101), between the *XbaI* sites, was
replaced with *EcoRI* linkers, resulting in plasmid
pAS203.   The *EcoRI* to *HindIII* fragment of pCMVβ
5   (CLONETECH) was rendered blunt ended and cloned in the
Klenow treated *EcoRI* site of pAS203 to yield pAAVlacZ.

Example 2
Construction of the pGN1909 Helper Plasmid

10   The AAV helper construct pGN1909 which is
capable of expressing the AAV Rep and Cap polypeptide
products includes an approximately 4.8 Kb nucleotide
stretch comprising an AAV *rep* and *cap* coding region
and a downstream AAV p5 promoter that are interposed
15   between two FRT sites.   The pGN1909 plasmid can be
constructed as follows.   Referring to Figure 1, a
BglII site is introduced 12 bases 5' of the *rep78/68*
ATG in the previously-described pAAV/Ad construct
(Samulski et al. (1989) *J. Virol.* 63:3822-3828)
20   resulting in a plasmid called pAAV/Ad-*Bgl*.   A 50 bp
minimum FRT site (Jayaram (1985) *Proc. Natl. Acad.*
Sci. USA 82:5875-5879; and O'Gorman (1991) *Science*
251:1351-1355) is then inserted into the *BglII* site of
pAAV/Ad-*Bgl* and the resulting FRT-rep-cap fragment
25   lacking the p5 promoter is cloned into the polylinker
of pBSII k/s- (Stratagene, San Diego, CA) to yield a
plasmid called pFRTRepCap.
         In a separate step, a fragment defined by
the *SpeI* and *PstI* sites of the previously described
30   pIM29+45 plasmid (McCarty et al. (1991) *J. Virol.*
65:2936-2945) which includes an AAV p5 promoter and a
portion of the 5' end of the *rep* gene is subcloned
into the polylinker of pBSII k/s- between the *SpeI* and
PstI sites.   An FRT site is then introduced 12 bp 5'
35   of the *rep78/68* ATG, resulting in a plasmid called
pGN1901.   The *SpeI-PstI* fragment of pGN1901 is
inserted into the *XbaI* site of the pFRTRepCap plasmid

resulting in the pGN1909 plasmid construct. A map of
the pGN1909 plasmid is depicted in Figure 2.

A similar construct can be constructed as
follows. The following five nucleotide fragments,
5   arranged in the order given in the 5' to 3' direction
are ligated into the polylinker of pBSII k/s- between
the *NotI* and the *PstI* sites: (1) a first nucleotide
fragment comprising the 59 bp "minimum" FRT site
(Jayaram and O'Gorman, *supra*) depicted in Figure 3
10   [SEQ ID NO:2]; (2) a second nucleotide fragment
comprising an AAV *rep* and *cap* coding region
(corresponding to bps 310 through 4484 of the wt AAV
genome, Srivastava et al. (1983) *J. Virol.* 45:555-
564); (3) a third nucleotide fragment comprising an
15   AAV p5 promoter region corresponding to bps 145
through 310 of the wt AAV genome (depicted in Figure 4
[SEQ ID NO:4]); (4) a fourth nucleotide fragment
comprising the 76 bp FRT site (Jayaram and O'Gorman,
supra) depicted in Figure 3 [SEQ ID NO:3]; and (5) a
20   fifth nucleotide fragment comprising a 184 bp segment
of the 5' end of the *rep* coding region corresponding
to bps 310 through 494 of the wt AAV genome (depicted
in Figure 4 [SEQ ID NO:4]).

The five nucleotide sequences are linked
25   together to form a single, contiguous nucleic acid
molecule having a synthetic junction interposed
between fragments 2 and 3 to facilitate the linkage of
those two moieties. The nucleotide sequence of the
synthetic junction is as follows: 5'—CTCTAGTGGATCT—3'
30   [SEQ ID NO:1]. Such junctions can be any suitable
linker moiety known in the art and are merely used
herein to facilitate the assembly of the subject AAV
helper construct.

35

Example 3
Construction of the pW1909 Helper Plasmid

The AAV helper plasmid pW1909, which is
capable of providing essential AAV Rep and Cap
5   polypeptides for recombinant AAV virion production,
generally comprises a 1909 AAV helper sequence (AAV
rep and *cap* coding regions having an AAV p5 promoter
arranged downstream therefrom). The pW1909 plasmid
was constructed from a pW1909adhLacZ plasmid
10   (described below) by cleaving the plasmid with
*Sse*8387, isolating the 6506 bp *Sse*8387I-*Sse*8387I
fragment and recircularizing the fragment by
intramolecular ligation.

The pW1909adhLacZ plasmid was constructed as
15   follows. The plasmid pUC119 (GeneBank Reference Name:
U07649, GeneBank Accession Number: U07649) was
partially digested with *Afl*III and *Bsp*HI, blunt-end
modified with the klenow enzyme, and then ligated to
form a circular 1732 bp plasmid containing the
20   bacterial origin and the *amp* gene only (the polylinker
and F1 origin was removed). The blunted and ligated
*Afl*III and *Bsp*HI junction forms a unique *Nsp*I site.
The 1732 bp plasmid was cut with *Nsp*I, blunt-end
modified with T4 polymerase, and a 20 bp
25   *Hin*DIII-*Hin*CII fragment (blunt-end modified with the
klenow enzyme) obtained from the pUC119 polylinker was
ligated into the blunted *Nsp*I site of the plasmid.
The *Hin*DIII site from the blunted polylinker was
regenerated, and then positioned adjacent to the
30   bacterial origin of replication. The resulting
plasmid was then cut at the unique *Pst*I/*Sse*8387I site,
and an *Sse*8387I-*Pvu*II-*Sse*8387I oligonucleotide
(5'—GGCAGCTGCCTGCA—3' [SEQ ID NO:5]) was ligated in.
The remaining unique *Bsp*HI site was cut, blunt-end
35   modified with klenow enzyme, and an oligonucleotide
containing an *Asc*I linker (5'—GAAGGCGCGCCTTC—3' [SEQ

-42-

ID NO:6]) was ligated therein, eliminating the *BspHI* site. The resulting plasmid was called pWee.

A CMVlacZ expression cassette was inserted into the unique *PvuII* site of pWee using multiple steps such that the CMV promoter was arranged proximal to the bacterial *amp* gene of pWee. The CMVlacZ cassette contains a nucleotide sequence flanked 5' and 3' by AAV ITRs. The nucleotide sequence has the following elements: a CMV promoter; the hGH 1st intron; an adhlacZ fragment; and an SV40 early polyadenylation site.

The plasmid psub201 (Samulski et al. (1987) *J. Virol* 61:3096-3101) was cut with *XbaI*, blunt-end modified, and *NotI* linkers (5'—TTGCGGCCGCAA—3') [SEQ ID NO:7] were ligated to the ends to provide a vector fragment containing the bacterial origin of replication and an *amp* gene, wherein the vector fragment is flanked on both sides by *NotI* sites. After being cut with *NotI*, the first CMV expression cassette was cloned into the psub201 vector fragment to create psub201CMV. The ITR-bounded expression cassette from this plasmid was isolated by cutting with *PvuII*, and ligated to pWee after that plasmid was cut with *PvuII* to create pWCMV. pWCMV was then cut with *BssHII* (partial), and a 3246 bp fragment containing the adhlacZ gene (a *SmaI-DraI* nucleotide fragment obtained from the plasmid pCMV-β, having *AscI* linkers (5'—GAAGGCGCGCCTTC—3') [SEQ ID NO:6] ligated to the ends to provide a 3246 bp fragment) was ligated into the *BssHII* site of pWCMV to obtain the pWadhlacZ construct.

Plasmid pW1909adhlacZ was obtained by combining elements from pGN1909 with the pWadhlacZ construct. In particular, a 4723 bp *SpeI-EcoRV* fragment containing the AAV *rep* and *cap* encoding region was obtained from the plasmid pGN1909. The 4723 bp fragment was blunt-end modified, and *AscI*

linkers were ligated to the blunted ends. The
resultant fragment was then ligated into the unique
AscI site of pWadhlacZ and oriented such that the AAV
coding sequences were arranged proximal to the
5   bacterial origin of replication in the construct.

Example 4
Production of Recombinant AAV Virions

Human 293 cells (Graham et al. (1967) *J.*
10   *Gen. Virol.* <u>36</u>:59-72, available from the ATCC under
Accession Number CRL1573) are grown in sterile DME/F12
culture medium (without HEPES buffer) that has been
supplemented with 10% fetal calf serum (FCS), 1%
pen/strep and 1% glutamine (Sigma, St. Louis, MO) at
15   37 °C in 5% CO_2. Once the cells are healthy and
dividing, they are trypsinized and plated at from 1 X
10^6 to 5 X 10^6 cells per 10 cm cell culture plate. A
monolayer confluency of 50 to 75% is achieved when the
cells initially attach to the surface of the plate.
20   The volume of medium in each plate is 10 mL.
Avoidance of any clumping of the cells and an even
distribution in the cell is essential in order to
achieve even cell density over all areas of the tissue
culture plate (which is important for high rAAV
25   particle yield). The cells are then grown at 37 °C in
5% CO_2 to reach 90% confluency over a period of from
24 to 48 hours before transfection.

At 1 to 4 hours prior to transfection, the
medium in the tissue culture plates is replaced with
30   fresh DME/F12 culture medium containing 10% FCS, 1%
pen/strep and 1% glutamine. 10μg each of the pAAVlacZ
vector and the pGN1909 helper construct (or another
suitable helper construct) are added to 1 mL of
sterile 300 mM $CaCl_2$, which is then added to 1 mL of
35   sterile 2X HBS solution (formed by mixing 280 mM NaCl,
50 mM HEPES buffer, 1.5 mM Na_2HPO_4 and adjusting the pH
to 7.1 with 10 M NaOH) and immediately mixed by gentle

inversion. The resultant mixture is then pipetted
immediately into the 10 cm plates of 90% confluent 293
cells (in 10 mL of the above-described culture medium)
and swirled to produce a homogeneous solution.

5   The plates are transferred to a 5% CO_2
incubator and cultured at 37 °C for 6 to 8 hours
without disturbing. After transfection, the medium is
removed from the plates, and the monolayer of cells
washed once with sterile Phosphate buffered saline

10 (PBS).

Adenovirus working stock is prepared by
diluting a master stock of adenovirus (serotype 2) to
a concentration of 10^6 pfu/mL in DME/F12 plus 10% FCS,
1% pen/strep, 1% glutamine and 25 mM sterile HEPES

15 buffer (pH 7.4). 10 mL of the resulting adenovirus
working stock is added to each 10 cm plate and the
cells are incubated for approximately 72 hours. When
all of the cells show cytopathic effect (CPE), and
approximately 30% of the cells are floating, the cells

20 are harvested by gently pipetting the cells to detach
them from the plate surface. The cell suspension is
collected and centrifuged at 300 x g for 2 minutes.
The supernatant is aspirated off and the cells are
resuspended in 1 mL of sterile Tris buffered saline

25 (TBS, prepared by mixing 100 mL Tris HCL, 150 mM NaCl
and adjusted to pH 8.0). The resultant material can
be frozen at -80 °C, or used immediately to make a
freeze/thaw lysate.

The freeze/thaw lysate is prepared by

30 freezing and thawing the TBS:cell suspension 3 times
by alternating between a dry ice/ethanol bath (until
the cells are completely frozen) and a 37 °C water
bath (until completely thawed). Tissue debris is
removed by centrifugation at 10,000 x g for 10

35 minutes. The supernatant is collected and transferred
to a sterile cryo-vial. The adenovirus is heat
inactivated by incubating the freeze/thaw lysate at 56

°C for 1 hour by submersion in a water bath. Any
precipitate that forms during the heat inactivation is
removed by centrifuging the sample at 10,000 x g for
10 minutes. The supernatant containing recombinant

5   AAV virions is then harvested. The virions can be
stored frozen at -70°C.

        Transducing vector titers can be determined
by infecting 293 cells with a dilution series of the
rAAV virions prepared above. After 24 hours, the

10   cells are fixed and stained with X-Gal (Sanes et al.
(1986) *EMBO* 5:3133-3142). The titer is calculated by
quantifying the number of blue cells.

                          Example 5
15   Comparison of AAV Helper Plasmid Efficiency
        The efficiency of helper functions provided
by the pGN1909 construct was compared against the
previously described pAAV/Ad and pIM29+45 AAV helper
plasmids (Samulski et al. and McCarty et al., *supra*).

20   rAAVlacZ virions were prepared by co-transfection of
human 293 cells with pAAVlacZ (prepared as described
above in Example 1) and one of the following three
helper plasmids: pGN1909, pAAV/Ad and pIM29+45. The
titers of recombinant preparations from the three

25   preparations were determined (as described above in
Example 4). The results are depicted in Table 1.

                          TABLE 1

AAV Helper Construct	Titers of rAAVlacZ
pGN1909	2.7×10^8/mL
pAAV/Ad	3.8×10^7/mL
pIM29+45	1.9×10^7/mL

        As can be seen by the above results, the
35   titers of recombinant preparations produced using the
pGN1909 construct as an AAV helper construct are 5 to

10 fold greater than preparations using either of the
two previously described helper plasmids.

<u>Example 6</u>
5   <u>Preparation of Elongated 1909 AAV Helper Sequences</u>
        It has been demonstrated that during
recombinant AAV virion production, unwanted
recombination events may occur between AAV vector and
AAV helper DNAs that result in the production of
10   replication competent AAV wild-type virus (wherein AAV
helper fragments have acquired AAV ITRs). In order to
reduce or eliminate wild-type AAV virus generation, an
intervening nucleotide sequence (X) was inserted into
the pW1909 helper construct between the AAV *cap* coding
15   region and the nucleotide sequence containing the AAV
p5 promoter. The intervening sequence (X) was
selected to have a length (ℓ) which renders wild-type
recombinants too large to be encapsidated as AAV
virions.
20   In particular, a set of four helper plasmids
was derived from pW1909 by inserting four
progressively longer intervening nucleotide sequences
(X1-X4) into the AAV helper sequence between the
polyadenylation site (3' of the AAV *cap* coding region
25   in the construct) and the AAV p5 promoter sequence.
The pW1909 plasmid was partially digested with
*Ecl*136II at position 5060. The X1 nucleotide sequence
(a 603 bp fragment obtained from phi X174,2) was
ligated into the *Ecl*136II site of pW1909 to obtain
30   pW1909-0.6. With the addition of the 603 bp X1
fragment, the pW1909-0.6 construct contains a 5349 bp
AAV helper sequence. The X2 nucleotide sequence (a
1078 bp fragment obtained from phi X174,3) was ligated
into the *Ecl*136II site of pW1909 to obtain pW1909-1.
35   With the addition of the 1078 bp X2 fragment, the
pW1909-1 construct contains a 5824 bp AAV helper
sequence. The X3 nucleotide sequence (a 2027 bp

fragment obtained from phage lambda) was ligated into
the *Ecl*136II site of pW1909 to obtain pW1909-2. With
the addition of the 2027 bp X3 fragment, the pW1909-2
construct contains a 6773 bp AAV helper sequence. The
5   X4 nucleotide sequence (a 4361 bp fragment obtained
from phage lambda) was ligated into the *Ecl*136II site
of pW1909 to obtain pW1909-4. With the addition of
the 4361 bp X4 fragment, the pW1909-4 construct
contains a 9107 bp AAV helper sequence.

10   The addition of two minimal (145 bp) AAV ITR
sequences to any of the elongated AAV helper sequences
of pW1909-0.6, pW1909-1, pW1909-2, or pW1909-4 would
result in AAV sequence lengths of 5639 bp, 5824 bp,
6773 bp, and 9107 bp, respectively. In this manner,
15 the size of wild-type recombinants generated from any
of those AAV helper constructs exceeds the AAV
packaging limit of about 5100 bp.

        The pW1909-0.6, pW1909-1, pW1909-2 and
pW1909-4 constructs were assessed for their ability to
20 provide enhanced AAV helper activity in rAAV virion
production. The pW1909 helper construct and the
pAAV/Ad helper plasmid were used as controls.

        AAV helper activity was assessed as follows.
rAAVlacZ virions were prepared by co-transfection of
25 human 293 cells (5 X 10^6 cells per 10 cm dish) with
pAAVlacZ (prepared as described above in Example 1)
and one of the following helper plasmids: pAAV/Ad or
pW1909 (as controls); pW1909-0.6; pW1909-1; pW1909-2;
or pW1909-4. rAAV virion production was conducted as
30 described above in Example 4, with the exception that
viral helper functions (normally provided by
adenoviral infection) were provided in the form of a
plasmid. More particularly, the 293 cell cultures
were co-transfected with 5 μg of each of the following
35 vectors: the pAAVlacZ vector; a selected AAV helper
plasmid; and a third plasmid containing selected
adenoviral genes or gene regions (E2a, E4 and VA RNA)

which supplied the adenoviral helper functions. The
cells were incubated for approximately 72 hours.
Preparation of freeze/thaw lysates and determination
of rAAVLacZ titers from the preparations were then
conducted as described in Example 4. The results are
depicted in Table 2.

TABLE 2

AAV Helper Construct	AAV Helper Sequence Length (bp)	rAAVlacZ Titer	% of pW1909
pAAV/Ad	4391	7×10^8	24
pW1909	4746	3×10^9	100
pW1909-0.6	5349	1×10^9	37
pW1909-1	5824	2×10^9	60
pW1909-2	6773	1×10^9	32
pW1909-4	9107	8×10^8	25

As can be seen, the titers of recombinant
preparations produced using the pW1909 helper
construct are four fold greater than preparations
obtained using the previously described "conventional"
helper plasmid pAAV/Ad. The 1909 constructs having
elongated AAV helper sequences (pW1909-0.6, pW1909-1,
pW1909-2 and pW1909-4) provided varying amounts of
virions, wherein pW1909-1 exhibited helper activity
that was substantially 60% as efficient as pW1909.

Example 7
Preparation of Modified Versions
of the pW1909 Helper Plasmid

Two modified versions of the pW1909 helper
plasmid, pH4 and pH8, were provided as follows.
Initially, an AAV helper plasmid, pUC4391, was
assembled by inserting a nucleotide sequence
containing base pairs 146-4530 of the AAV serotype 2
genome (Srivastava et al. (1983) *J. Virol.* 45:555-564)

into the SmaI site of pUC119. The plasmid was
arranged such that SmaI sites flank a 4391 bp
nucleotide fragment which contains all of the AAV
serotype 2 genome sequences except for the two 145
5   base pair ITR sequences. In particular, the 4567 bp
SmaI fragment obtained from pSM620 (Samulski et al.
(1982) Proc. Natl. Acad. Sci. USA 79:2077-2081) was
subcloned into the SmaI site of pUC119 (GeneBank
Reference Name: U07649, GeneBank Accession Number:
10   U07649) to provide pUC4567. M13-based mutagenesis was
then used to eliminate all AAV ITR sequences and
thereby provide pUC4391. The following mutagenic
oligonucleotides were used in the M13 mutagenesis:
5'—AGCTCGGTACCCGGGCGGAGGGGTGGAGTCG—3'; and
15   5'—TAATCATTAACTACAGCCCGGGGATCCTCTA—3'.
        The polylinker of pBSII k/s- (Stratagene,
San Diego) was removed by cutting with BssHII and
replaced with the following synthetic polylinker:
5'—GCGCGCCGATATCGTTAACGCCCGGGCGTTTAAACAGCGCTGGCGCGC—3'
20   [SEQ ID NO:8]. The synthetic polylinker contains the
following restriction sites: BssHII; EcoRV; HpaI;
SrfI; PmeI; Eco47III; and BssHII. The resulting
construct was termed "bluntscript."
        The 4399 bp SmaI fragment from pUC4391 was
25   cloned into the SrfI site of bluntscript such that the
5' end of the rep coding region of the AAV helper
sequence was proximal to the HpaI site of the
synthetic polylinker. The resulting plasmid was
termed pH1. The AAV p5 promoter region and AAV rep 5'
30   untranslated sequence of pH1 was replaced with a
promoterless 5' untranslated sequence obtained from
the pW1909 helper plasmid. In particular, the 323 bp
BssHII(blunted)-SrfI fragment from pW1909 was cloned
into a 7163 bp SrfI-SmaI(partial) fragment obtained
35   from pH1 to obtain pH2. The pH2 construct was used to
provide the two AAV helper plasmids, pH4 and pH8,
which contain two different versions of an AAV p5

promoter region arranged at the 3' end of the AAV
helper sequence.

    In particular, the pH4 helper plasmid was
constructed by cloning the 727 bp *BglI-PstI*(blunted)
5   fragment from pW1909 into the *Eco*47III site of pH2.
The 727 bp sequence from pW1909 contains an AAV p5
promoter region, an FRT site and a portion of the N-
terminal *rep* coding sequence. The direction of
transcription from the 3' AAV p5 promoter region is
10   the same as the direction of transcription of the AAV
p19 and AAV p40 promoter regions.

    The pH8 helper plasmid was constructed by
inserting a 172 bp nucleic acid fragment containing
the AAV p5 promoter region into the *Eco*47III site of
15   pH2. Thus, the pH8 helper plasmid does not have an
FRT recombination site or additional AAV nucleotide
sequences associated with the 3' AAV p5 promoter
region. Further, the 3' AAV p5 promoter region
transcribes in the same direction as the AAV p19 and
20   AAV p40 promoter regions.

    The efficiency of helper functions provided
by the pH4 and pH8 helper plasmids in the production
of rAAVlacZ virion production was compared against the
pW1909 plasmid. rAAV virion production was conducted
25   as described above in Example 4, with the exception
that viral helper functions (normally provided by
adenoviral infection) were provided in the form of a
plasmid. More particularly, the 293 cell cultures
were co-transfected with 5 μg of each of the following
30   vectors: the pAAVlacZ vector; a selected AAV helper
plasmid; and a third plasmid containing selected
adenoviral genes or gene regions (E2a, E4 and VA RNA)
which supplied the adenoviral helper functions. The
cells were incubated for approximately 72 hours.
35   Preparation of freeze/thaw lysates and determination
of rAAVLacZ titers from the preparations were then

conducted as described in Example 4. The results are depicted in Table 3.

TABLE 3

AAV Helper Construct	FRT Site Location	3' AAV p5 Promoter	rAAVlacZ Titer
pW1909	5' and 3'	1909	1×10^8
pH4	5' and 3'	1909	2×10^8
pH8	5' only	minimal	2×10^8

As can be seen, both pH4 and pH8 have approximately the same activity as the pW1909 helper plasmid, indicating that deletion of the 3' FRT site and 3' AAV *rep* sequences did not adversely affect the function of the pW1909-derived, pH8 helper plasmid.

Deposits of Strains Useful in Practicing the Invention

A deposit of biologically pure cultures of the following strains was made with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, under the provisions of the Budapest Treaty. The accession number indicated was assigned after successful viability testing, and the requisite fees were paid. Access to said cultures will be available during pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR 1.14 and 35 USC 122. All restriction on availability of said cultures to the public will be irrevocably removed upon the granting of a patent based upon the application. Moreover, the designated deposits will be maintained for a period of thirty (30) years from the date of deposit, or for five (5) years after the last request for the deposit; or for the enforceable life of the U.S. patent, whichever is longer. Should a culture become nonviable or be inadvertently destroyed, or, in the case of

plasmid-containing strains, lose its plasmid, it will
be replaced with a viable culture(s) of the same
taxonomic description.

These deposits are provided merely as a

5   convenience to those of skill in the art, and are not
an admission that a deposit is required. The nucleic
acid sequences of these plasmids, as well as the amino
sequences of the polypeptides encoded thereby, are
controlling in the event of any conflict with the

10 description herein. A license may be required to
make, use, or sell the deposited materials, and no
such license is hereby granted.

	Strain	Deposit Date	ATCC No.
15	pGN1909	July 20, 1995	69871

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-53-

Claims

1.   A nucleic acid molecule encoding AAV
helper functions, said molecule comprising:

5   an AAV *rep* coding region and an AAV *cap*
coding region, said AAV *rep* and *cap* coding regions
operably linked to control sequences that direct the
expression thereof; and
          a first nucleotide sequence comprising an

10   AAV p5 promoter region, said first nucleotide sequence
arranged in the molecule such that the p5 promoter
region is situated in a site that is other than its
natural position relative to the *rep* coding region in
the wild-type AAV genome.

15

2.   The nucleic acid molecule of claim 1
wherein the first nucleotide sequence is substantially
homologous to base pairs 145 through 309 as depicted
in Figure 4 [SEQ ID NO:4] of the wild-type AAV

20   serotype 2 genome.

3.   The nucleic acid molecule of claim 1
wherein the first nucleotide sequence is substantially
homologous to base pairs 145 through 494 as depicted

25   in Figure 4 [SEQ ID NO:4] of the wild-type AAV
serotype 2 genome.

4.   The nucleic acid molecule of claim 1
further comprising a second nucleotide sequence that

30   is homologous to a Flip Recombination Target (FRT)
site.

5.   The nucleic acid molecule of claim 1
further comprising a 5' and a 3' flanking nucleotide

35   sequence, wherein each said flanking sequence is
substantially homologous to an FRT site and further
wherein the AAV *rep* coding region, the AAV *cap* coding

region, and the nucleotide sequence comprising the AAV
p5 promoter region are interposed between said
flanking nucleotide sequences.

5   6.   The nucleic acid molecule of claim 1
further comprising an intervening nucleotide sequence,
wherein the molecule comprises, in the order given in
the 5' to 3' direction, an AAV *rep* coding region, an
AAV *cap* coding region, the intervening nucleotide
10   sequence, and a nucleotide sequence comprising an AAV
p5 promoter region.

           7.   The nucleic acid molecule of claim 6
wherein the intervening nucleotide sequence has a
15   length sufficient to prevent the packaging in an AAV
virion of a molecule produced by a recombination of
said nucleic acid molecule with AAV ITRs during
recombinant AAV virion production.

20   8.   The nucleic acid molecule of claim 7
wherein the intervening nucleotide sequence comprises
at least 500 base pairs.

           9.   An AAV helper construct comprising the
25   nucleic acid molecule of claim 1.

           10.   The AAV helper construct of claim 9
wherein said construct comprises a plasmid.

30   11.   The AAV helper construct of claim 10
wherein said construct comprises the plasmid pGN1909
(ATCC Accession Number 69871).

           12.   An AAV helper construct comprising the
35   nucleic acid molecule of claim 4.
           13.   The AAV helper construct of claim 12
wherein said construct comprises a plasmid.

14. The AAV helper construct of claim 13 wherein said construct comprises the plasmid pH8.

15. An AAV helper construct comprising the nucleic acid molecule of claim 7.

16. The AAV helper construct of claim 15 wherein said construct comprises a plasmid.

17. The AAV helper construct of claim 16 wherein said construct comprises the plasmid pW1909-1.

18. The construct of claim 9 further comprising a selectable genetic marker.

19. The construct of claim 18 wherein the selectable genetic marker comprises an antibiotic resistance gene.

20. An AAV packaging cell prepared by transfecting a suitable host cell with the AAV helper construct of claim 9.

21. An AAV packaging cell prepared by transfecting a suitable host cell with the AAV helper construct of claim 12.

22. An AAV packaging cell prepared by transfecting a suitable host cell with the AAV helper construct of claim 15.

23. An AAV producer cell capable of producing recombinant AAV virions when viral helper functions are expressed therein, said producer cell comprising the packaging cell of claim 20 that has been transfected with an AAV vector.

24.   An AAV producer cell capable of
producing recombinant AAV virions when viral helper
functions are expressed therein without the
concomitant production of significant levels of wild-
5   type AAV, said producer cell comprising the packaging
cell of claim 21 that has been transfected with an AAV
vector.

25.   An AAV producer cell capable of
10   producing recombinant AAV virions when viral helper
functions are expressed therein without the
concomitant production of significant levels of wild-
type AAV, said producer cell comprising the packaging
cell of claim 22 that has been transfected with an AAV
15   vector.

26.   A method of producing recombinant AAV
virions, comprising the steps:
        (a)   introducing an AAV vector into a host
20   cell;
        (b)   introducing the AAV helper construct of
claim 9 into the host cell such that the construct
provides essential AAV Rep and Cap polypeptides;
        (c)   expressing viral helper functions in
25   the host cell; and
        (d)   culturing the cell to produce
recombinant AAV virions.

27.   The method of claim 26 wherein the
30   expression of viral helper functions is provided by
infecting the host cell with a virus selected from the
group consisting of adenovirus, herpesvirus and
vaccinia virus.

35   28.   A method of producing recombinant AAV
virions, comprising the steps:

(a) introducing an AAV vector into a host cell;

(b) introducing the AAV helper construct of claim 12 into the host cell such that the construct provides essential AAV Rep and Cap polypeptides;

(c) expressing viral helper functions in the host cell; and

(d) culturing the cell to produce recombinant AAV virions.

29.   The method of claim 28 wherein the expression of viral helper functions is provided by infecting the host cell with a virus selected from the group consisting of adenovirus, herpesvirus and vaccinia virus.

30.   A method of producing recombinant AAV virions without the concomitant production of significant levels of wild-type AAV, comprising the steps:

(a) introducing an AAV vector into a host cell;

(b) introducing the AAV helper construct of claim 15 into the host cell such that the construct provides essential AAV Rep and Cap polypeptides;

(c) expressing viral helper functions in the host cell; and

(d) culturing the cell to produce recombinant AAV virions.

31.   The method of claim 30 wherein the expression of viral helper functions is provided by infecting the host cell with a virus selected from the group consisting of adenovirus, herpesvirus and vaccinia virus.
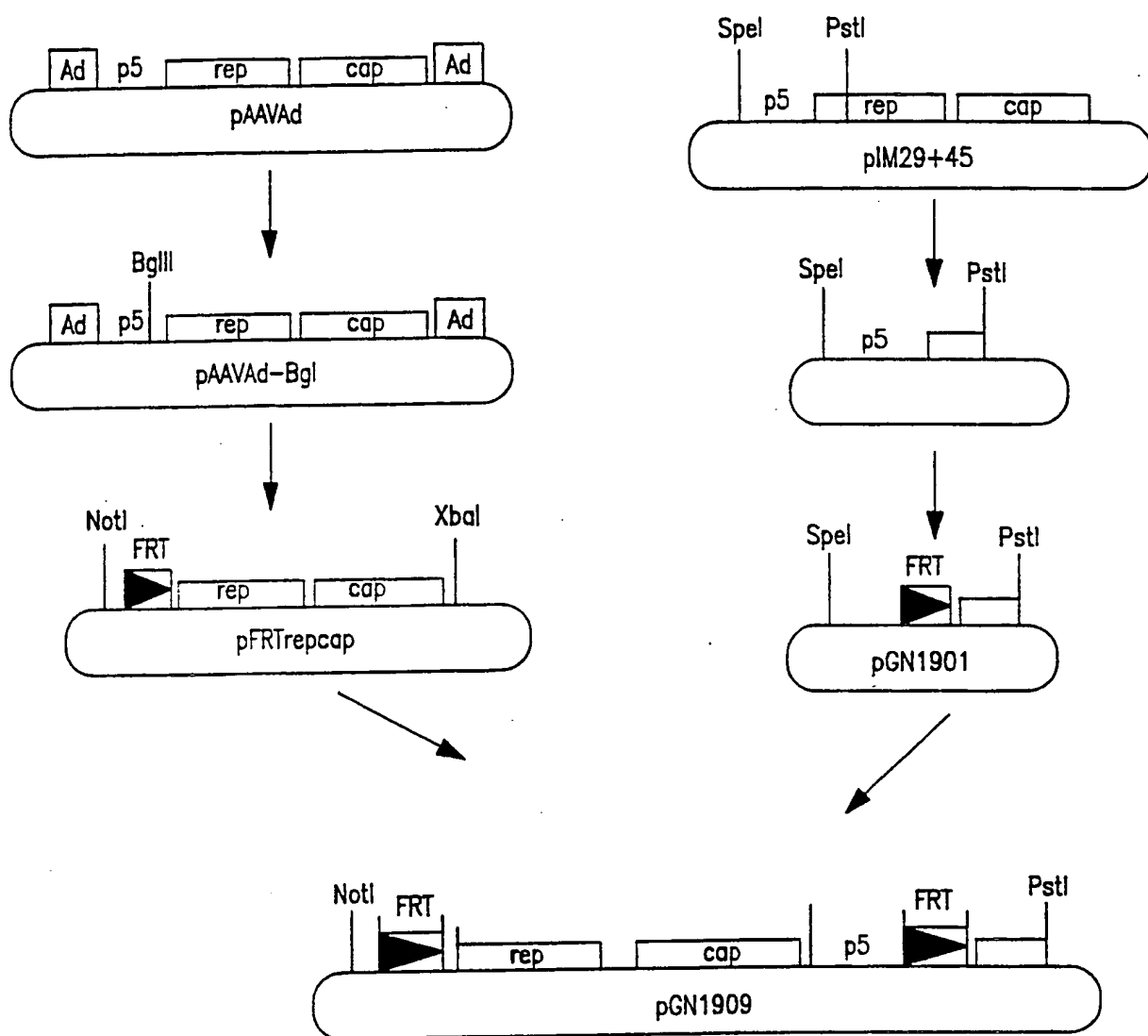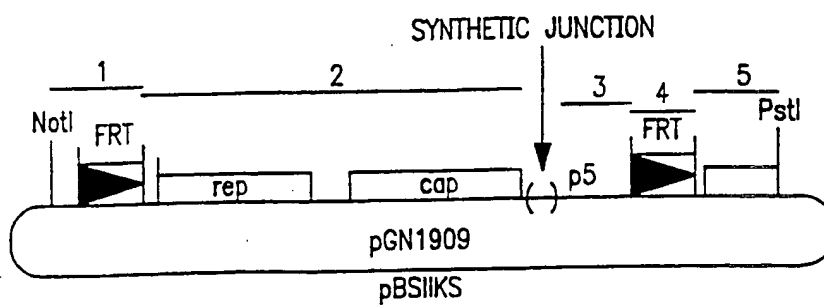
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FIG. 1

FIG. 2

(59 bp "minimum" FRT site):

GATCAGAAGT TCCTATTCCG AAGTTCCTAT TCTCTAGAAA
GTATAGGAAC TTCTGATCT

(76 bp FRT site):

GATCAGAAGT TCCTATTCCG AAGTTCCTAT TCTCTAGAAA
GTATAGGAAC TTCAGAGCGC TTTTGAAGCT CTGATC

FIG.3

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  1                                     GGAGG GGTGGAGTCG

 16   TGACGTGAAT TACGTCATAG GGTTAGGGAG GTCCTGTATT AGAGGTCACG

 66   TGAGTGTTTT GCGACATTTT GCGACACCAT GTGGTCACGC TGGGTATTTA

116   AGCCCGAGTG AGCACGCAGG GTCTCCATTT TGAAGCGGGA GGTTTGAACG

166   CGCAGCCGCC ATGCCGGGGT TTTACGAGAT TGTGATTAAG GTCCCCAGCG

216   ACCTTGACGG GCATCTGCCC GGCATTTCTG ACAGCTTTGT GAACTGGGTG

266   GCCGAGAAGG AATGGGAGTT GCCGCCAGAT TCTGACATGG ATCTGAATCT

316   GATTGAGCAG GCACCCCTGA CCGTGGCCGA GAAG
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FIG.4